

ORIGINAL PAPER

Infection with the roundworm *Toxocara canis* leads to exacerbation of experimental allergic airway inflammation

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Clinical and Experimental Allergy

Summary

Background Epidemiological studies performed in developing as well as in western countries suggest that infection with *Toxocara canis* contributes to the development of atopic diseases. **Objectives** To investigate the association between infection with this helminth and allergy, we examined the effect of *T. canis* infection on experimental allergic airway inflammation. **Methods** BALB/c mice were infected by oral administration with 500 embryonated *T. canis* eggs followed by ovalbumin (OVA) sensitization and challenge to induce allergic airway inflammation.

Results Infection with *T. canis* in combination with OVA treatment leads to exacerbation of pulmonary inflammation, eosinophilia, airway hyperresponsiveness, OVA specific and total IgE. Relative quantification of cytokine expression in the lungs of these mice showed increased expression of IL-4 compared with mice that were only *T. canis* infected or OVA treated. Increased expression of IL-5 and IL-10 was measured in the lungs of *T. canis*-infected or OVA-treated mice compared with controls; however, combining infection and OVA treatment did not significantly change the expression of these cytokines.

Conclusion A previous infection with *T. canis* leads to exacerbation of experimental allergic airway inflammation. These results have important consequences for findings on the helminths–allergy association. Several factors, including parasite species, infection of definitive vs. accidental host, parasite load and timing of infection, may influence whether an infection with helminths protects one from or enhances allergic manifestations.

Keywords airways, cytokines, experimental allergic airway inflammation, hyper-reactivity, mice, *Toxocara canis*

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Introduction

Toxocara canis is a roundworm of dogs that also infects humans world-wide [1]. Infected animals pass *T. canis* eggs in their faeces, which they deposit in playgrounds, sandpits, backyards, etc. Children and adults become infected after ingesting the infectious eggs while playing in these recreation areas, gardening or carrying out any other activity dealing with *T. canis*-contaminated soil. After ingestion of infectious eggs, larvae penetrate the intestinal wall and migrate to different organs, including the lungs. Humans are accidental hosts and the larvae will not mature to the adult stage but they migrate throughout the body and can survive for many years [2].

Seroprevalence surveys have shown that human exposure to *Toxocara* varies with age and geographical area. The exposure to *Toxocara* spp. in the Netherlands based on serological surveys has been reported to be 19% on average, with 4–15% in people younger than 30 years, and 30% for the age group older than 45 years [3]. In the state of Connecticut, USA, seroprevalence varied from 6.1% in New Haven to 27.9% in Bridgeport [4] and in Nepal an average seroprevalence of 81% was reported [5].

Although usually asymptomatic, infection with this helminth can lead to the syndrome known as visceral larva migrans characterized by several clinical signs, including coughing, wheezing and mucus production [6].

From several epidemiological studies it has been suggested that infection with *T. canis* contributes to the development of allergic manifestations, including asthma (reviewed in [7]). The majority are cross-sectional studies from children [8, 9] and adult [10] populations in which *Toxocara* exposure is defined by the presence of antibodies in serum directed to *T. canis* excretory/secretory antigens. In one study, Buijs et al. [8] evaluated the relationship between *Toxocara* seroprevalence and allergic asthma in schoolchildren aged 4–6 years. The authors found that occurrences of asthma/recurrent bronchitis, and hospitalization due to asthma/recurrent bronchitis, were significantly associated with *Toxocara* seropositivity. The authors suggest that *Toxocara* worms may contribute to the manifestation of allergic asthma in children predisposed to allergy.

Previous studies using murine models for toxocariasis have shown that infection with *T. canis* results in persistent pulmonary inflammation, eosinophilia, IgE production, airway hyper-reactivity and production of T-helper type 2 (Th2) cytokines [11–13]. In these studies, infection with this helminth has been suggested to be associated with the enhancement of allergic manifestations. However, this association has not been directly addressed. The present study is to our knowledge the first that investigates the effect of infection with *T. canis* on allergic manifestations by combining a murine model for toxocariasis and the experimental model for allergic airway inflammation. The effect of timing of infection on allergic airway inflammation was also investigated.

Materials and methods

Parasites

T. canis eggs were collected from the uteri of female worms as previously described [12]. Briefly, collected eggs were allowed to embryonate in 0.1 M H₂SO₄ in the dark at room temperature for 4–6 weeks and were stored in 0.1 M H₂SO₄ at 4 °C until use.

Experimental design

Specified pathogen-free female BALB/c mice (5–6 weeks) were obtained from Harlan Netherlands BV (Horst, the Netherlands). Mice were housed in Macrolon III cages with food and water *ad libitum*. This study included six groups of mice consisting of eight animals per group unless indicated otherwise. The animals were infected with *T. canis*, treated with ovalbumin (OVA) (allergic airway inflammation model) or both. The effect of timing of infection on allergic airway inflammation was investigated by exposing the mice to OVA treatment 3 or 20 days post-infection (p.i.). The number of larvae in the lungs of *T. canis*-infected mice has been shown to differ between 3

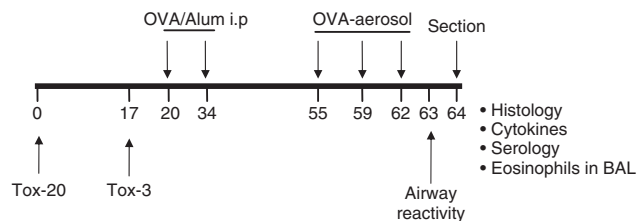


Fig. 1. Schematic representation of the experimental design. Mice were infected with 500 infectious *Toxocara canis* eggs and exposed to an ovalbumin (OVA) sensitization/challenge protocol at 3 or 20 days post-infection, as indicated in Materials and methods. One day before mice were killed measurement of airway reactivity was performed. Following post-mortem, cytokine gene expression and histology of the lungs was carried out in addition to quantification of eosinophils in bronchoalveolar lavage and determination of OVA-specific and total IgE in serum.

and 20 days p.i. At 3 days p.i. *T. canis* larvae have already reached the lungs and by 20 days p.i. they have migrated to other organs and eventually accumulated in the brain [14]. The groups consisted of mice infected on day 0 (Tox-20); on day 17 (Tox-3); infected with *T. canis* and 3 days treated with OVA (Tox-3+OVA); infected with *T. canis* and 20 days p.i. treated with OVA (Tox-20+OVA); OVA treated only (OVA) and control uninfected and untreated animals (Ctrl).

Mice from groups Tox-3 and Tox-3+OVA were killed 47 days p.i. Mice from groups Tox-20 and Tox-20+OVA and Ctrl were killed 64 days p.i. Infection was performed by oral administration of 500 embryonated eggs per mouse in 0.3 mL sterile phosphate-buffered saline (PBS). The egg suspension was administered orally to the mice using a syringe fitted with a blunt needle. Treatment with OVA consisted of 42 days in total with initial sensitization by two intraperitoneal (i.p.) immunizations with 10 µg OVA (grade V, Sigma-Aldrich, Steinheim, Germany) adsorbed onto 2.25 mg aluminium hydroxide in 100 µL saline solution (OVA/Alum) administered to the mice with 14 days in between. Twenty-one days after the last immunization, a challenge was performed by exposing the mice for 30 min with OVA-aerosol (10 µg/mL) in saline generated by a nebulizer (Ultra-NebTM2000; DeVilbiss, Langen, Germany). The challenge with OVA was repeated 4 and 3 days later (Fig. 1). Control uninfected/untreated mice received 0.3 mL saline orally, 100 µL alum i.p. and saline during aerosol challenge. All experiments were performed according to international and institutional guidelines for animal care.

Histology of the lungs

Mice were anaesthetized 2 days after the last OVA challenge by i.p. injection of a mixture of Ketalar (Park and Davis, Hoofddorp, the Netherlands) and Rompun (Bayer, Leverkusen, Germany). Mice were killed by exsanguination via the abdominal aorta. The lungs were subsequently

perfused via the right cardiac ventricle by injection of 10 mL PBS and thereafter removed. The left lung was frozen in liquid nitrogen and later used for RNA isolation. The right lung was fixed with 10% neutral-buffered formalin and embedded in paraplast (Kendall, Tyco Healthcare, Boston, MA, USA). Transverse sections of 5 µm were stained with haematoxylin-eosin and with periodic acid Schiff for mucus staining. Histological changes (peribronchiolitis, perivascular infiltrate, hypertrophy of goblet cells, alveolitis and eosinophil influx) were semi-quantitatively and blindly scored from absent (0), minimal (1), slight (2), moderate (3), marked (4) to severe (5) as previously reported [12].

Ovalbumin-specific immunoglobulin E

Determination of OVA-specific IgE levels was performed using a capture ELISA. Microtitre 96-well plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 2.0 µg/mL of rat-anti-mouse IgE (Invitrogen, Carlsbad, CA, USA) in sodium carbonate buffer, 0.05 M, pH 9.6 (Sigma, St Louis, MO, USA). After four washes with PBS supplemented with 0.1% Tween 20 (Sigma), a blocking step was performed by incubating the wells with blocking buffer: 0.05 M Tris-buffered saline with bovine serum albumin 1% (w/v), pH 8.0 (Sigma) for 1 h at 37 °C, or overnight at 4 °C. After the plates were washed four times, serum samples were diluted in sample buffer (blocking buffer supplemented with 0.05% Tween 20) and added to the plate for 1 h at 37 °C in a twofold serial dilution starting at 1 : 80. In addition, duplicate twofold dilutions of an OVA-specific IgE reference serum (made from i.p. OVA immunized mice) were also added, with a 1 : 10 starting dilution, in order to create a standard curve. Plates were washed and incubated for 1 h at 37 °C with a 1 : 4000 dilution of digoxigenin (DIG)-conjugated OVA made with a protein labelling kit (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. After washing, the plates were incubated for 2 h at 37 °C with a 1 : 625 dilution of anti-DIG FAb fragments coupled to horseradish peroxidase (Roche). For colour development tetramethylbenzidine (TMB) was used as substrate. The reaction was stopped with an equal volume of a 10% (v/v) H₂SO₄ solution (Merck, Darmstadt, Germany). The absorbance was read at 450 nm and results were expressed as arbitrary units (AU), where the lowest dilution of the reference serum was set to 1000 AU.

Total immunoglobulin E

Total serum IgE antibodies were measured in sera by using a capture ELISA (OptEIA™ Set) according to the manufacturer's recommendations (PharMingen, San Diego, CA, USA). Briefly, plates were coated overnight at 4 °C with the capture antibody (anti-mouse IgE) diluted in coating

buffer. After blocking each well, standard samples and controls were added. The working detector solution (biotinylated anti-mouse IgE antibody and peroxidase-conjugated streptavidin) was then added. Finally the colour development was performed using TMB as substrate and the absorbance read at 450 nm. Total IgE concentration was calculated from the standard titration curve using the purified mouse IgE provided by the OptEIA™ Set. The detection limit of the total IgE assay is 1.6 ng/mL.

Analysis of bronchoalveolar lavage cells

Lungs were lavaged three times with 1 mL PBS (pH 7.2, 37 °C). The bronchoalveolar lavage (BAL) was centrifuged at 400 × g, at 4 °C, for 10 min and the cell pellet was resuspended in 1 mL PBS. Total cell number was determined by mixing 0.5 mL of the cell suspension with 9.5 mL isoton and using a cell counter (Beckman Coulter BV, Mijdrecht, the Netherlands). From the cell suspension, cytopins were made and differential cell counts were performed after the preparations were stained with May-Grunwald/Giemsa. At least 400 cells/slide were counted by light microscopy. The number of eosinophils in BAL is expressed as eosinophils × 10⁶ in BAL (cells/mL) ± SD.

Airway responsiveness

Airway responsiveness was measured in conscious, unrestrained mice using whole-body plethysmography (BUXCO, EMKA Technologies, Paris, France) as described previously [12]. Briefly, mice were placed in individual chambers; the baseline readings were taken and averaged for 3 min. Animals were then exposed to aerosolized PBS or increasing concentrations of methacholine (Sigma) from 2 to 7.5 mg/mL during 3 min. After nebulization the readings were taken and averaged for 3 min. Results are expressed as Penh (enhanced pause) ± SE at 7.5 mg/mL methacholine concentration.

Real-time reverse-transcriptase polymerase chain reaction

Total RNA extraction from lungs using TRIzol reagent (Invitrogen) and cDNA synthesis was performed as previously described [15]. IL-4, IL-5, IL-10 and IFN-γ mRNA transcripts were quantified, relative to the house-keeping gene, β-actin, using the Roche LightCycler® FastStart DNA Master^{PLUS} SYBR Green I kits (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. Briefly, the reaction mixture consisted of a master mix containing Taq DNA polymerase, dNTP mixture and SYBR Green I, 24 mM MgCl₂, 10 pmol of each primer and 2 µL of cDNA template in 20 µL. For validation, plasmids containing the relevant cloned cytokine genes were used as standards and were included in each PCR run. A ratio between the values of the target gene and the

house-keeping gene was performed as described previously [15]. The normalized mRNA levels in samples from infected/treated mice were then expressed relative to levels in control mice, and are reported as the mean percentage increase over controls.

Statistical analysis

The statistical difference between groups was determined by a one-way ANOVA using SPSS for Windows version 12.0. Results from the histological examinations were statistically compared using the Wilcoxon test. A probability value of $P < 0.05$ was considered to be statistically significant in all cases.

Results

Histology of the lungs

Table 1 shows the pathological changes observed in the lungs of BALB/c mice infected with 500 *T. canis* embryonated eggs and/or OVA treatment. Results indicate that mice infected with *T. canis* only (Tox-3 and Tox-20) showed a slight peribronchiolar and moderate perivascular infiltrate consisting of mononuclear cells, lymphocytes and eosinophils. The hypertrophy of the bronchiolar mucous cells was moderate to marked. Alveolitis was slight and consisted of polymorphonuclear cells (PMNs), eosinophils and large macrophages containing granular material. The pathological lesions were not significantly different between groups Tox-3 and Tox-20.

Mice treated with OVA to induce allergic airway inflammation (OVA) showed a slight peribronchiolar and moderate perivascular infiltrate (mononuclear cells and eosinophils) together with a moderate hypertrophy of the bronchiolar mucous cells. In some animals a minimal alveolitis was present.

Mice that were infected with *T. canis* for 3 days (Tox-3+OVA) or 20 days (Tox-20+OVA) and then treated with OVA showed increased pathology compared with mice that were only infected with *T. canis* (Tox-3 and Tox-20) or with the OVA-treated group of animals (Figs 2a–d). Lungs of mice from groups Tox-3+OVA and Tox-20+OVA showed a moderate peribronchiolitis, while the perivascular infiltrate was severe, with eosinophils representing the main infiltrating cell. The hypertrophy of the bronchiolar mucous cells was also severe. The moderate to marked alveolitis consisted of eosinophils, large macrophages and PMNs. The lesions observed in the lungs of mice from groups Tox-3+OVA and Tox-20+OVA were not significantly different. In the control group (Ctrl) no histological lesions were observed.

Total immunoglobulin E in bronchoalveolar lavage and serum

The concentration of total IgE in BAL from mice of all experimental groups is shown in Fig. 3a. Infection of mice with 500 *T. canis* eggs (Tox-3 and Tox-20) resulted in a significant increase of total IgE in BAL compared with uninfected/untreated controls. Treatment of mice with OVA only also resulted in significant increased total IgE in BAL compared with the Ctrl group. The combination of infection followed by the OVA sensitization/challenge protocol resulted in a significant increase of total IgE compared with mice that were infected only or received OVA treatment only. The concentration of total IgE in BAL from Tox-3+OVA mice was 4.2 times higher compared with Tox-3 mice, and 8.6 times higher compared with OVA mice. Tox-20+OVA mice had total IgE concentrations in BAL 8.8 times higher compared with Tox-20 mice, and 12.3 times higher compared with OVA mice. No significant difference was found between the total IgE concentration in BAL of mice from groups Tox-3+OVA and Tox-

Table 1. Pathological changes in the lungs of BALB/c mice infected with 500 *Toxocara canis* embryonated eggs and exposed to OVA-treatment

	Tox-3	Tox-3+OVA	Tox-20	Tox-20+OVA	OVA	Ctrl
Peribronchiolitis	1.8 ± 0.4	2.8 ± 0.4 ^{*,**}	2.0 ± 0.0	2.6 ± 0.5	1.7 ± 0.5	0
Perivascularitis	3.5 ± 0.5	4.8 ± 0.4 ^{*,**}	3.3 ± 0.5	5.0 ± 0.0 ^{*,***}	2.7 ± 0.5	0
Hypertrophy mucous cells	3.7 ± 0.5	4.7 ± 0.5 ^{*,**}	3.7 ± 0.5	4.8 ± 0.4 ^{*,***}	3.2 ± 0.4	0
Alveolitis	2.5 ± 1.0	3.7 ± 0.8 [*]	2.0 ± 0.0	2.8 ± 0.4 ^{*,***}	0.3 ± 0.5	0
Eosinophils influx	3.0 ± 0.0	3.7 ± 0.5 [*]	3.2 ± 1.0	3.6 ± 0.5 [*]	1.5 ± 0.5	0

Transverse lung sections were performed in *T. canis* infected mice (Tox-3, Tox-20), mice that were *T. canis* infected and at 3 or 20 days post-infection treated with OVA (Tox-3+OVA, Tox-20+OVA), mice with OVA treatment only (OVA) and the control (Ctrl) uninfected/untreated mice. Data are mean of histological scores of six mice/group.

^{*}Significantly different ($P < 0.05$) from group OVA.

^{**}Significantly different ($P < 0.05$) from group Tox-3.

^{***}Significantly different ($P < 0.05$) from group Tox-20. The histopathological score was: absent (0), minimal (1), slight (2), moderate (3), marked (4) to severe (5).

BAL, bronchoalveolar lavage; OVA, ovalbumin.

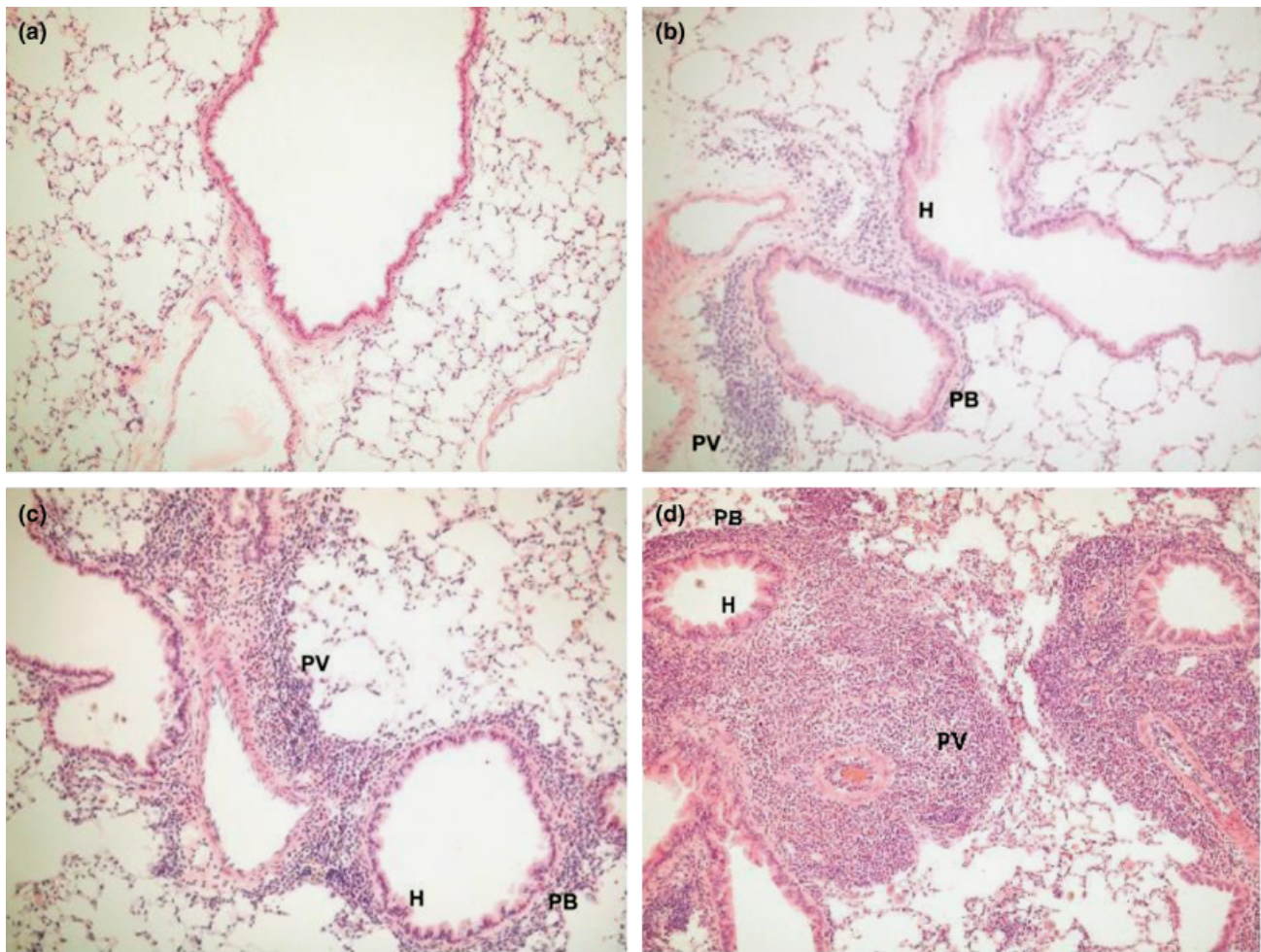


Fig. 2. (a–d). Histological sections of lung tissues from BALB/c mice after infection with 500 *Toxocara canis* embryonated eggs and/or ovalbumin (OVA) treatment. (a) Lung of control uninfected/untreated mouse with a bronchiole (centre, top), covered with a thin epithelium and an accompanying vessel without infiltrate. Alveoli are clean and open. Stained with haematoxylin–eosin (HE), $\times 160$. (b) Lung of a mouse treated with OVA. Bronchioles are covered with hypertrophied mucous cells (H), evaluated as moderate. Peribronchiolitis (PB) and perivascularitis (PV) is slight. HE, $\times 160$. (c) Lung of a mouse, infected with *T. canis* (Tox-3). The bronchiole is covered by moderately hypertrophied epithelium, and surrounded by moderate PB and PV. HE, $\times 160$. (d) Lung of mouse, infected with *T. canis* and treated with OVA (Tox-3+OVA). The hypertrophy (H) of the bronchiolar epithelium (top left and right) is evaluated as severe. The PV is severe, mainly consisting of polymorphonuclear cells. The PB is moderate and the alveoli contain moderate number of inflammatory cells (alveolitis moderate). HE, $\times 160$.

20+OVA. Comparable results were found in the sera of these animals (Fig. 3b). The average concentration of total IgE in the sera of Tox-3+OVA and Tox-20-OVA mice was respectively 3.9 and 5.2 times higher compared with the OVA group. The total IgE concentration was 2.3 times higher in the Tox-3+OVA group compared with the Tox-3 group, and was 3.1 times higher in the Tox-20+OVA group compared with the Tox-20 mice.

Ovalbumin-specific immunoglobulin E

The levels of OVA-specific IgE in the sera of OVA-treated mice with or without a *T. canis* infection are shown in Fig. 4. Animals from the Tox-20+OVA group showed significant higher OVA-specific IgE levels compared with

the Tox-3+OVA group and with mice that received OVA treatment only. The level of OVA-specific IgE in the sera of mice from the Tox-20+OVA group was 1.6 times higher compared with the OVA group. There was no significant difference between the Tox-3+OVA and OVA groups.

Eosinophils in bronchoalveolar lavage

The number of eosinophils in BAL from mice after infection with *T. canis* with or without the OVA treatment and uninfected/untreated controls is shown in Fig. 5. Results indicate that infection with 500 embryonated *T. canis* eggs (groups Tox-3 and Tox-20) or treatment with OVA resulted in a significant increased number of eosinophils in BAL compared with uninfected/untreated control

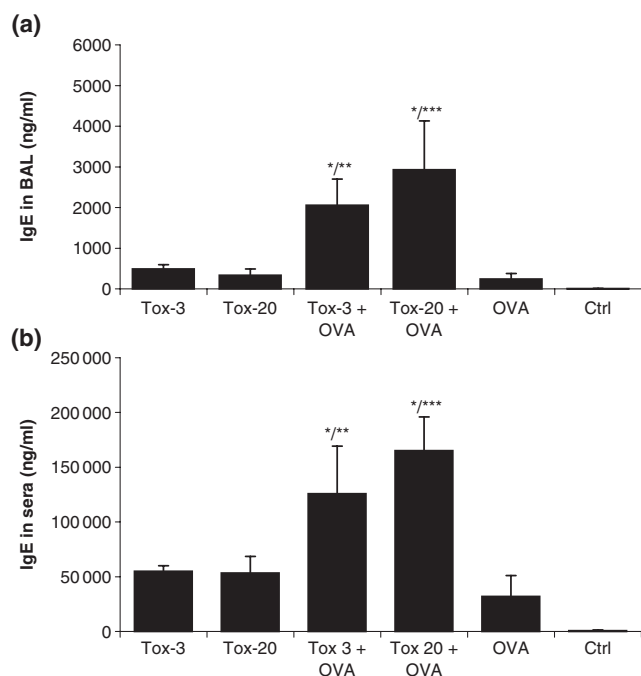


Fig. 3. (a,b) Total IgE concentration in (a) bronchoalveolar lavage (BAL) or (b) sera of BALB/c mice after infection with *Toxocara canis* with or without the ovalbumin (OVA) sensitization/challenge protocol. Total IgE concentration was measured in BAL and sera from *T. canis*-infected mice (Tox-3, Tox-20), *T. canis*-infected mice treated with OVA at 3 or 20 days post-infection (Tox-3+OVA, Tox-20+OVA), OVA treated only mice (OVA) and the control (Ctrl) uninfected/untreated mice. Data are mean of IgE concentration of eight mice/group expressed as ng/mL $\pm$ SD. *Significantly different ($P < 0.05$) from group OVA. **Significantly different ($P < 0.05$) from group Tox-3. ***Significantly different ($P < 0.05$) from group Tox-20.

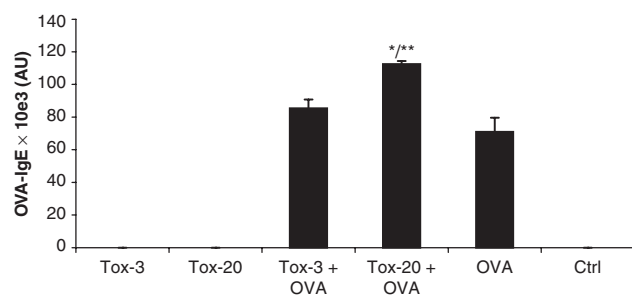


Fig. 4. Ovalbumin (OVA)-specific IgE in sera of BALB/c mice after infection with *Toxocara canis* with or without the OVA sensitization/challenge protocol. The OVA-specific IgE concentration was measured in sera from *T. canis* infected mice (Tox-3, Tox-20), mice that were *T. canis* infected and at 3 or 20 days post-infection treated with OVA (Tox-3+OVA, Tox-20+OVA), mice with OVA treatment only (OVA) and the control (Ctrl) uninfected/untreated mice. Data are mean of OVA-IgE concentration of eight mice/group expressed as arbitrary units (AU) $\pm$ SD. *Significantly different ($P < 0.05$) from OVA group. **Significantly different ($P < 0.05$) from group Tox-3+OVA.

animals. The combination of infection with *T. canis* followed by OVA treatment (groups Tox-3+OVA and Tox-20+OVA) resulted in a significant increase in the

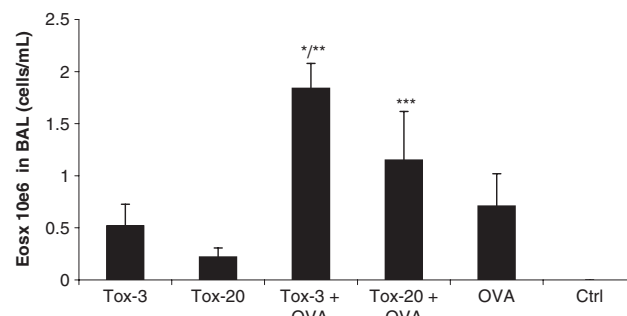


Fig. 5. Eosinophils in bronchoalveolar lavage (BAL) of BALB/c mice after infection with *Toxocara canis* with or without the ovalbumin (OVA) sensitization/challenge protocol. Analysis of cells in BAL was performed for *T. canis* infected mice (Tox-3, Tox-20), mice that were *T. canis* infected and at 3 or 20 days post-infection treated with OVA (Tox-3+OVA, Tox-20+OVA), mice with OVA treatment only (OVA) and the control (Ctrl) uninfected/untreated mice. Data are mean of eosinophils (Eos) in BAL from eight mice/group and are expressed as eosinophils (Eos) $\times 10^6$ in BAL (cells/mL) $\pm$ SD. *Significantly different ($P < 0.05$) from group OVA. **Significantly different ($P < 0.05$) from group Tox-3. ***Significantly different ($P < 0.05$) from group Tox-20.

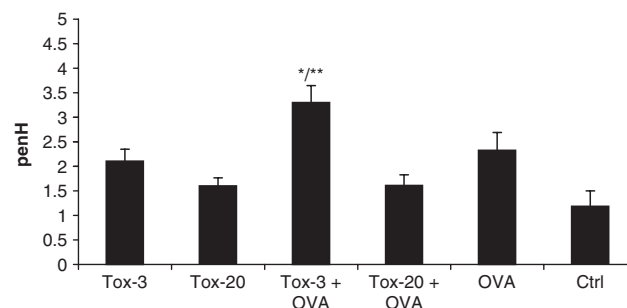


Fig. 6. Airway responsiveness of BALB/c mice after exposure to 7.5 mg/mL methacholine. Reactivity of the airways was measured in *Toxocara canis* infected mice (Tox-3, Tox-20), mice that were *T. canis* infected and at 3 or 20 days post-infection treated with ovalbumin (OVA) (Tox-3+OVA, Tox-20+OVA), mice with OVA treatment only (OVA) and the control (Ctrl) uninfected/untreated mice. Results are from six mice/group and are expressed as Penh $\pm$ SD. *Significantly different ($P < 0.05$) from group OVA. **Significantly different ($P < 0.05$) from group Tox-3.

number of eosinophils in BAL compared with mice that were *T. canis* infected or OVA treated only.

Airway reactivity

Airway reactivity was measured in conscious, unanesthetized mice after exposure to increasing doses of methacholine. The airway responsiveness, expressed as Penh, was measured in uninfected/untreated animals and in mice that were infected with *T. canis*, treated with OVA, or a combination of both. Figure 6 shows the Penh values at 7.5 mg/mL methacholine for different groups of animals. The results indicate that the reactivity of the airways for the Tox-3+OVA group was significantly higher than

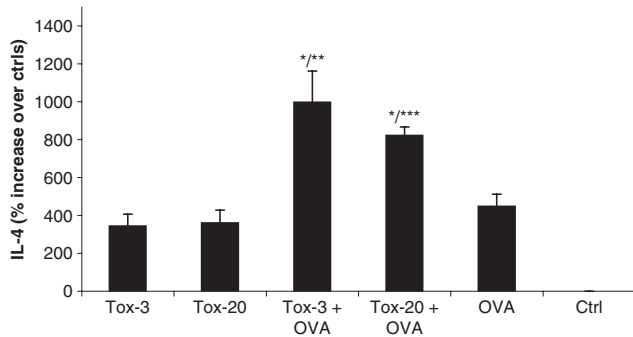


Fig. 7. Relative quantification of IL-4 levels in lungs of *Toxocara canis*-infected BALB/c mice with or without ovalbumin (OVA) treatment. The normalized mRNA levels of IL-4 in lungs from *T. canis* infected mice (Tox-3, Tox-20), mice that were *T. canis* infected and at 3 or 20 days post-infection treated with OVA (Tox-3+OVA, Tox-20+OVA), and mice with OVA treatment only (OVA) were expressed relative to levels of the IL-4 in control uninfected/untreated mice (Ctrl), and are reported as the mean percentage increase ($\pm$ SD) over controls. *Significantly different ($P < 0.05$) from group OVA. **Significantly different ($P < 0.05$) from group Tox-3. ***Significantly different ($P < 0.05$) from group Tox-20.

that of the OVA and Tox-3 group of animals. No significant difference in reactivity of the airways was observed for mice from the Tox-20 and Tox-20+OVA groups compared with the uninfected/untreated control mice.

Cytokine expression in lungs

Real-time RT-PCR assays were used for the relative quantification of mRNA encoding for IL-4, IL-5, IL-10 and IFN- γ in the lungs of mice infected with 500 embryonated *T. canis* eggs and/or treated with OVA. The results indicate that compared with uninfected/untreated controls, the expression of IL-4 increases after *T. canis* infection, OVA treatment or a combination of both (Fig. 7). Furthermore, the expression of the IL-4 transcript was significantly higher in the lungs of mice that received a *T. canis* infection followed by OVA treatment (Tox-3+OVA and Tox-20+OVA) compared with animals that were only infected (Tox-3 and Tox-20) or treated with OVA. The expression levels of IL-5 and IL-10 significantly increased after infection or OVA treatment; however, combining these two (Tox+OVA) did not significantly change the expression of these cytokine genes. Expression of IFN- γ did not differ significantly between the different groups of animals.

Discussion

The association between helminth infections and atopic diseases has interested researchers for many years. This interest is mainly because these two disorders share immunological features such as a dominant Th2 type of immune response that is characterized by increased serum

IgE and eosinophilia [16]. Remarkably, certain helminth infections have been reported to suppress [17–20] while others to contribute to the development of allergic manifestations [10, 21–24].

Although *T. canis* does not develop to the adult stage in humans, tissue-dwelling larvae could remain for many years in the body, infecting several organs including the lungs [1]. Clinical manifestations such as mucus production, wheezing and coughing that are normally observed in patients with allergic asthma are also characteristic of individuals infected with *Toxocara* spp. Evidence from epidemiological studies carried out both in western and in developing countries have shown that allergic manifestations occur more often in *Toxocara* sero-positive individuals, suggesting that infection with this helminth contributes to the development of allergic manifestations [8, 9, 21–25]. In one study, however, neither a positive nor a negative correlation between *Toxocara* sero-positivity and allergic manifestations was found [4].

Similar to humans, mice can also be infected with *T. canis* and the larvae migrate throughout the body without reaching the adult stage. Several studies using murine models have shown that infection with this helminth results in pulmonary inflammation, mucus production and hyper-reactivity of the airways [11, 15, 26], which is also observed in patients with visceral larval migrans due to a *T. canis* infection [6, 27]. In these studies *Toxocara* infections have been suggested to enhance inflammatory responses in allergic patients; however, this association has not been directly investigated.

In order to investigate the effect of *T. canis* infection on allergic manifestations, we combined a murine model for toxocariasis and an experimental model for allergic airway inflammation. We observed that a single infection with 500 infectious *T. canis* eggs results in exacerbation of experimental allergic airway inflammation, corroborating the findings from the epidemiological studies [8, 9, 21–25].

Several factors may influence the association between helminth infections and allergic manifestations. These include the helminth species involved; whether the host is a definitive host or an accidental host in which the parasite does not develop to the adult stage; the frequency of infection; the parasite load and the timing of infection in relation to allergen exposure. To address the latter possibility, we infected mice with *T. canis* either 3 or 20 days before the OVA immunization challenge protocol. At 3 days p.i. *T. canis* larvae have already reached the lungs and by 20 days p.i. the number of larvae decreases [14], although they do not disappear completely because previous studies have shown a small number of larvae still present in the lungs of BALB/c mice at 60 days p.i. [12]. We observed exacerbation of total IgE in serum and BAL, pulmonary pathology and IL-4 gene expression in the lungs of mice from Tox-3+OVA and Tox-20+OVA groups

compared with animals that were *T. canis* infected or OVA treated only. The difference in timing of infection, which is related to the number of larvae present in the lungs at the time mice received the OVA treatment, affected the reactivity of the airways and the number of eosinophils in BAL, because these parameters were higher only in the Tox-3+OVA group compared with the Tox-20+OVA, Tox-3, Tox-20 and OVA groups. The production of OVA-specific IgE was also affected by the timing of infection in relation to OVA treatment because significantly higher values were found only in the Tox-20+OVA group compared with the Tox-3+OVA and OVA groups.

Airway reactivity was measured at 47 days after infection for mice from groups Tox-3 and Tox-3+OVA and at 64 days after infection for groups Tox-20 and Tox-20+OVA. We have previously shown that infection with 500 *T. canis* embryonated eggs leads to hyper-reactivity of the airways that persists up to 30 days p.i. [12]. At 60 days p.i. the reactivity of the airways was not different from that of the control uninfected mice. In the present study, as expected, no airway hyper-reactivity was observed in mice from the Tox-20 group and the treatment with OVA did not affect this result. In contrast, the combination of infection and OVA treatment 3 days later resulted in a significant increase in airway reactivity compared with all the other groups of animals. These results indicate that the timing of infection, indicating in this case a higher number of larvae in the lungs followed by OVA treatment, is crucial in affecting airway reactivity.

Concerning the relative quantification of cytokine expression, we found significant increased amounts of IL-4 mRNA in the lungs of *T. canis*+OVA mice compared with OVA-treated only or *T. canis*-infected only groups of animals. IL-4 is a cytokine that mediates IgE production [28]; therefore, the increased expression of this cytokine gene is in accordance with the increased production of total IgE measured both in BAL and in the sera of these mice.

Infection with *T. canis* followed by OVA treatment also had an effect on the number of eosinophils in BAL, with significantly higher values for the Tox-3+OVA group compared with animals that were only *Toxocara* infected or OVA treated. IL-5 mediates eosinophilia [29], and although we measured increased expression of IL-5 in the lungs of *Toxocara*-infected or OVA-treated mice, the expression of this cytokine did not change when mice were infected and then treated with OVA (data not shown). This discrepancy could be due to the timing at which samples were taken to measure the expression of this cytokine. Additional studies such as the measurement of cytokines produced by lung cells and characterization of the kinetics of cytokine expression in the lungs of *Toxocara*-infected mice should be carried out.

A positive correlation between infection and allergic manifestations is not limited to *Toxocara* infections only.

In 1981 Desowitz and colleagues reported the prevalence of antibodies to *T. canis* and *Dirofilaria immitis* in asthmatic and non-asthmatic children born and raised in Hawaii. They found a significantly higher prevalence of parasite-specific IgE antibodies in the asthmatic compared with the non-asthmatic population [30]. Palmer et al. [31] carried out a cross-sectional study in China and found an association between *Ascaris lumbricoides* infection and increased aero-allergen sensitization, airway inflammation and asthma. Recently, Hunninghake et al. [24] have examined the relationship between sensitization to *A. lumbricoides* and measures of asthma morbidity and severity in a Costa Rican population. The authors conclude that in areas with a low prevalence of helminthiasis, *Ascaris* sensitization may be an important marker of severe atopy and disease morbidity in children with asthma. Infection with this parasite is particularly interesting because for the same parasite a protective role against allergic manifestations has also been described [18, 32]. A possible explanation for these findings is the burden of infection in the different studied populations with low-grade infections being associated with enhancement of allergic reactivity and high-grade infection with protection [16]. Exacerbation of allergic asthma in children has also been reported for other pathogens such as respiratory syncytial virus (RSV) [33, 34]. Using experimental models, several authors have also shown that RSV infection enhances inflammatory responses in allergic mice, although it depends on timing of infection and prior immunization with the virus [35].

Although the protective effect of helminth infections on allergic manifestations has been extensively reported, also using experimental models [36–38], the mechanism by which these pathogens modulate the immune response and affect other diseases such as allergies is not fully understood. Emerging evidence indicates a role for Tregs induced during helminth infection in down-regulating immunopathologies such as experimental allergic airway inflammation [39]. It is suggested that induction of Tregs during helminth infections may protect the host against parasite-induced inflammation, and may thereby simultaneously cause down-regulation of excessive allergen-induced, inflammatory reactions [40]. Whether failure in the induction of functional Tregs in *Toxocara*-infected mice is the reason why instead of conferring protection, infection with this helminth enhances allergic manifestations still remains to be investigated.

Infection with helminths has been suggested to have therapeutic potential for different immunopathologies and, in fact, patients with inflammatory bowel diseases are already being deliberately infected with helminths [41]. In this study, however, we show that a previous infection with *T. canis* results in exacerbation of experimental allergic airway inflammation, corroborating previous epidemiological studies in humans. Currently, we

are investigating the effect of lower infective doses and repeated infections, which is most likely to occur in human populations, particularly in western countries. The mechanism by which helminths modulate the immune response and the identification of the molecules involved will be the focus of our future studies. The use of parasite molecules as a means for suppressing immunopathologies could be an alternative approach to the use of helminth infections because, as we demonstrate here, not all helminths have the ability to suppress inflammatory diseases.

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